Novel phosphate–phosphonate hybrid nanomaterials applied to biology

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Abstract

A new process for preparing oligonucleotide arrays is described that uses surface grafting chemistry which is fundamentally different from the electrostatic adsorption and organic covalent binding methods normally employed. Solid supports are

modified with a mixed organic/inorganic zirconium phosphonate monolayer film providing a stable, well-defined interface. Oligonucleotide probes terminated with phosphate are spotted directly to the zirconated surface forming a covalent linkage. Specific binding of terminal phosphate groups with minimal binding of the internal phosphate diesters has been demonstrated. On the other hand, the reaction of a bisphosphonate bone resorption inhibitor (Zoledronate) with calcium deficient hydroxyapatites (CDAs) was studied as a potential route to local drug delivery systems active against bone resorption disorders. A simple mathematical model of the Zoledronate / CDA interaction was designed that correctly described the adsorption of Zoledronate onto CDAs. The resulting Zoledronate-loaded materials were found to release the drug in different phosphate-containing media, with a satisfactory agreement between experimental data and the values predicted from the model.

Introduction

While research at the frontier between Chemistry and Biology is of major importance for the design of new drugs, there is also high potential for developing novel nanomaterials for biotechnology. In this context, metal phosphonate chemistry can be applied to the synthesis of biomaterials. Indeed, it is well known that phosphonic acids can react with various inorganic precursors, including metal salts or oxides or alkoxides, in organic or aqueous solution, leading to organic-inorganic hybrid networks [1].The key feature is the formation of metal-oxygen bonds, that can be highly covalent depending on the nature of the metal. Our idea was to modify inorganic surfaces (phosphonates, phosphates, oxides…) by coordination of the metal cations present on the surface, using phosphonic acids having various biological properties (Figure 1).

In a first example, we will report a new support for the preparation of DNA arrays, in which the biological probes are bound to a monolayer-coated surface through an "inorganic" linkage, in contrast to existing systems based on the attachment of the probes *via* organic bonding. A second example will describe a novel drug delivery system, based on the chemical grafting of a gem-bisphosphonate monolayer on various calcium phosphates.

Results and discussion

1. Metal phosphonates as novel supports for the preparation of oligonucleotide microarrays

DNA arrays have emerged as a convenient tool in molecular biological research, for rapid and accurate gene mapping, DNA sequencing, mRNA expression analysis, and diagnosis of genetic diseases [2,3]. Typical sensors consist either of

double-stranded products (PCR) or single-stranded oligonucleotides of different sequences, called probes, bound to a surface and amenable to subsequent hybridization by targets. A popular approach to microarrays involves “spotting” techniques that use automated robots to array oligonucleotides previously synthesized by chemical or enzymatic methods. Glass substrates are preferred for arrays, since it is flat and non-porous, and so hybridization volume can be kept to a minimum, and it is durable under the temperatures and chemical conditions normally employed. Furthermore, the low fluorescence of glass does not significantly contribute to background noise when fluorescence is used for detection. However, oligonucleotides bind poorly to glass, so some surface derivatization is required. The current trend is to form a surface bound monolayer of functional groups that are available to react only with a specific group on the probe terminus, resulting in covalent linkage. Organosilane-coating protocols are commonly used to fix the active groups to the glass surface (Figure 2). Some combinations of surface/oligonucleotide function that have been demonstrated include thiol/acrylamide [4], activated carboxylic acid/amine [5,6], amine/aldehyde [7-9], epoxide/amine [10], aldehyde/oxyamine [11].

Our purpose was to propose a fundamentally different route for covalently attaching DNA probes to surfaces for array applications. The new approach uses a mixed organic/inorganic monolayer to derivatize the glass and generate a reactive surface (Figure 3), using the Langmuir Blodgett (LB) technique. The LB process begins with an octadecylphosphonic acid (ODPA) Langmuir monolayer that is deposited onto the hydrophobic solid support in such a way that the hydrophilic acid group (PO_3H_2) is directed away from the support [12,13]. The substrate is then removed from the LB trough and exposed to a solution of Zr^{4+} ions that bind to give a

monolayer of the zirconated octadecylphosphonic acid (ODPA-Zr). In zirconium phosphonates, each Zr^{4+} ion coordinates to more than one phosphonate molecule and the phosphonates bind to more than one metal ion. Therefore, the extremely strong binding of the zirconium ions crosslinks the original monolayer and provides a stable, well-defined interface of zirconium phosphonate sites [12,13]. The ODPA-Zr monolayer sticks strongly to the surface because it is no longer a traditional LB film of individual molecules physisorbed to the surface, but rather a network or monolayer tape where adhesion comes from the sum of all molecules in a crosslinked array. The ODPA-Zr monolayers can be stored in water for months and retain activity with no evidence of desorption. The metal ions now on the surface are active and react readily when exposed to other phosphonic acids or organophosphates to bind them to the surface (Figure 1). For example, in a recent study, we used these surfaces to bind monolayers of a phosphorylated manganese porphyrin oxidation catalyst and studied organic transformations at the monolayer with no desorption of the film, permitting detailed studies of a surface-confined molecular catalyst [14].

The coordination properties of free phosphate (OPO_3H_2) are very close to those of the phosphonic acid function, and phosphate species graft similarly to zirconium phosphonate surfaces. As phosphate is a naturally occurring function that does not alter the intrinsic nature of the probe, and that can be introduced chemically or with enzymatic routes, we have used such nucleotides to build microarrays on the ODPA-Zr surfaces (Figure 4) using commercially available arrayers and scanners for the printing and imaging steps.

High specificity of the oligonucleotide anchoring onto the support has been demonstrated, along with good sensitivity of the resulting immobilized probes for detecting complementary targets [15]. Although not yet investigated, the methodology

should be readily adapted to iono-covalent anchoring of phosphorylated double-stranded DNA prepared by PCR, a process that is harder with the more common organic covalent anchoring mechanisms because they require non-natural modification of the probes. While not required, improved signal-to-noise ratio is obtained by post-spotting treatment with BSA (Bovine Serum Albumin) to passivate the unspotted regions of the arrays. Signal-to-noise ratios as high as 1000 have been demonstrated after hybridizing with fluorescent targets. The BSA treatment displaces physisorbed probes and protects the array from nonspecific binding of targets. The BSA also provides a biocompatible hydrophilic surface. Further improvements in performance were observed when the probe is linked to the terminal phosphate via a spacer. Oligomeric guanine spacers of 7-11 units were found to be optimal, while polyA, polyC, and polyT spacers did not show the same enhancement (Figure 5) [15,16].

2. Metal phosphonate-based drug delivery systems active against bone resorption disorders

It is easy to imagine that the idea of interfacing mixed organic/inorganic interfaces can be extended to other applications. The concept of using the coordinating abilities of phosphate or related phosphonic acid groups towards metal ions on inorganic surfaces can also be extended to other bio-technology problems. One example is the design of better drug delivery systems that could reduce side effects, improve efficacy of existing drugs and open the door to entire classes of new treatments. Given that the strength of the interaction between the phosphate or phosphonate and the metal support can be tuned by changing the nature of the metal center, the immobilization of therapeutic agents that naturally bear such functional groups onto inorganic biocompatible carriers could offer great potential in the field of

medical devices. In this context, calcium phosphate ceramics [CaPs], commonly used as implants for bone reconstruction [17,18] appear to be good candidates, since they can be resorbed by bone cells. For example, we were able to chemically combine CaPs with a geminal bisphosphonate (Zoledronate) [19,20] that is efficient for the treatment of post-menopausal osteoporosis [21] and bone metastases [22] (Figure 6). Indeed, we have shown that CDAs (calcium deficient apatites) undergo chemisorption of the drug through a surface adsorption process, due to PO_3 for PO_4 exchange (Figure 6) [19,20]. A simple mathematical model was designed, that correctly described the Zoledronate / CDAs interaction at equilibrium as Equation (1) (Figure 7), in simplified media such as ultrapure water or phosphate buffers [23]:

$$Z + P - X \equiv \underset{k-}{\overset{k+}{\rightleftarrows}} Z - X \equiv + P \qquad \text{Equation (1)}$$

where $X\equiv$ corresponds to the surface binding sites of the CDA that must be in interaction with either a Zoledronate (Z) or a phosphate (P) moiety.

It was noticed that for a given phosphate concentration and a given solid/liquid ratio, the Zoledronate concentration released in solution decreased for decreasing Γ bisphosphonate loading ratios. This suggests that the therapeutic effect (*i.e.* the amount of Zoledronate released in the medium) might be tuned according to the initial value of Γ. Furthermore, in spite of the complex composition of a culture medium, containing an important number of potentially competing species for Zoledronate, our simple model allowed to predict the Zoledronate release in simulated *in vitro* conditions. This clearly indicated that the phosphate concentration is the main parameter governing Zoledronate desorption when sorbed onto CDAs. The ability of the resulting biomaterials to release the bisphosphonate drug was demonstrated using *in vitro* studies [20], and in a recent *in vivo* experiment, we were able to establish the proof of concept for apatitic materials used as drug delivery systems. The local

bisphosphonate release from an apatitic calcium phosphate coating allowed to increase the mechanical fixation of an orthopedic implant (Figure 8) [24]. Obviously the increase in peri-implant bone density appeared to be dependent on the Zoledronate content of the coating. The potential of this type of biomaterial to stabilize newly formed bone tissue in osteoporotic implantation sites is currently under investigation.

Conclusion

In the present paper, we have described that inorganic surfaces can be modified by coordination of metal cations present on the surface, using phosphonic acids or closely related phosphate compounds, having various biological properties. The resulting organic-inorganic hybrid materials can be applied to biology. In a first example, we have reported a new approach for covalently immobilizing oligonucleotides on glass slides for microarray applications that takes advantage of mixed organic/inorganic zirconium phosphonate chemistry. On the other hand, we have shown that one type of gem-bisphosphonate, Zoledronate, a potent inhibitor for bone resorption, can be chemically associated onto calcium deficients apatites (CDAs). The ability of such materials to release Zoledronate suggests that they could be used as local bisphosphonate delivery systems, active against osteoporosis.

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Figure 1. Schematic representation of the modification of inorganic surfaces for the preparation of biologically active materials.

Figure 2. Scheme illustrating covalent immobilization of oligonucleotide probes, using organo-silane coating protocols.

Figure 3. Langmuir-Blodgett route to zirconated phosphonate modified slides. **a**. In step 1, a monolayer of octadecylphosphonic acid (ODPA) is deposited onto a hydrophobic slide (here, made hydrophobic with octadecyltrichlorosilane, OTS). In step 2, the slide is then exposed to a solution of $Zr^{4+}_{(aq)}$. The slide can then be rinsed and used immediately or stored in water for months before use. **b**. the zirconated ODPA slides are very smooth (right), compared here to the OTS covered glass before deposition (left).

Figure 4. Organic/inorganic surfaces for DNA arrays. Phosphate terminated probes attach specifically via coordinate covalent bonding (bonds are left out of the figure for clarity) to a zirconated phosphonic acid modified surface.

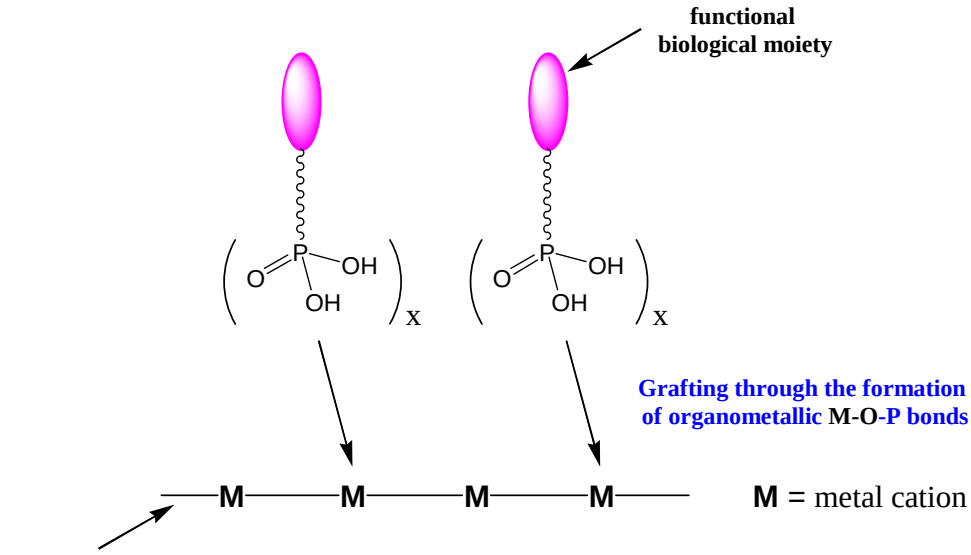
Figure 5. Fluorescence enhancement upon hybridization with labeled target related to the introduction of a polyG spacer to the probe. The effect is present for different probes (sequence X, Y or Z) spotted at different concentrations. The slides were spotted with **5'H_2O_3PO-$(B)_{11}$-O33** and hybridized with 100 nM complements **comp-5'CY3-O33,** where **O33** corresponds to a 33mer oligonucleotide (sequence X, Y or Z).

Figure 6.Schematic representation of the chemical association of Zoledronate onto CDAs (left). ^{31}P 2D SC14 Single Quantum-Double Quantum (SQ-DQ) NMR correlation spectrum obtained for a Zoledronate-loaded CDA(NaOH) sample [Γ = 0.97]. For clarity, the 2D data set is presented as a homonuclear SQ-SQ correlation (shearing transformation of the vertical DQ dimension). The ^{31}P 1D spectrum on the top is the projection of the SC14 2D spectrum on the horizontal SQ axis, that is fully similar to the ^{31}P Cross-Polarisation 1D spectrum obtained using the same contact time. Off-diagonal cross-peaks are identified by grey arrows. The 2D correlation spectrum shows clearly the presence of off-diagonal cross-correlation peaks between the Zoledronate and CDA resonances giving evidence of the spatial proximity between the phosphorus sites of the two compounds. The low intensity of these peaks is in agreement with a chemisorption process that only occurs on the surface of the CDA granules, thereby leaving the bulk of the phosphate groups away from Zoledronate.

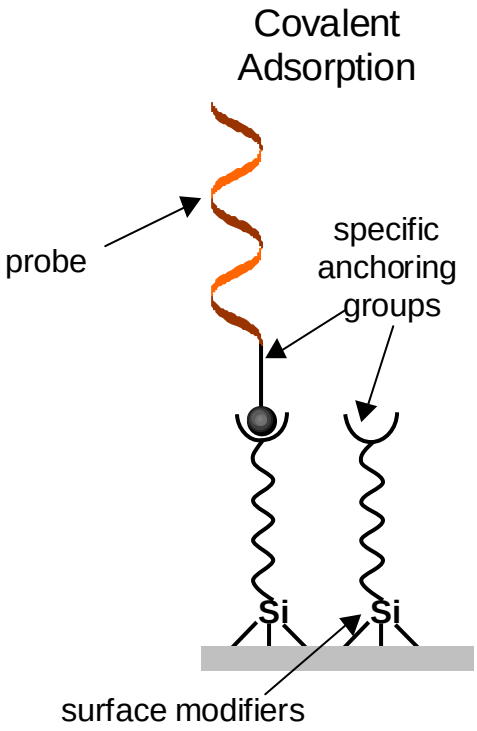
Figure 7. Zoledronate / CDA(NH_3) (filled symbols) or CDA(NaOH) (open symbol) interaction study of in phosphate buffers. (A): Sorption isotherms measured as a function of the Solid/Liquid ratio in the 0.25 to 200 g/L range. (B) Percentage of Zoledronate desorbed as a function of the phosphate concentration introduced in solution. (C) Zoledronate released as a function of the Zoledronate loading ratio Γ. $[P]_{tot}$ = 0.25 mol/L, S/L = 1 g/L. The lines correspond to calculated values according to Equation (1) (solid line: CDA(NH_3); dotted line: CDA(NaOH)). Zoledronate concentrations were analyzed by Total Organic Carbon (TOC) measurements or by liquid scintillation counting when ^{14}C-labeled Zoledronate was used. CDA(NH_3) and

CDA(NaOH) were obtained by alkaline hydrolysis of dicalcium phosphate dihydrate (DCPD), using aqueous ammonia or sodium hydroxide, respectively.

Figure 8. SEM pictures of two implanted rat condyles. Panel (a) shows the bone structure of a condyle implanted with a coated implant containing 2 μg Zoledronate (top) and the peri-implant bone (bottom), at magnification of 10X and 23X, respectively. Panel (b) shows the same views for a coated implant containing no Zoledronate.


functional
biological moiety
O
P
OH
OH
x
Grafting through the formation
of organometallic M-O-P bonds
M
M
M
M
M = metal cation
inorganic surface : oxide, phosphate, phosphonate....

1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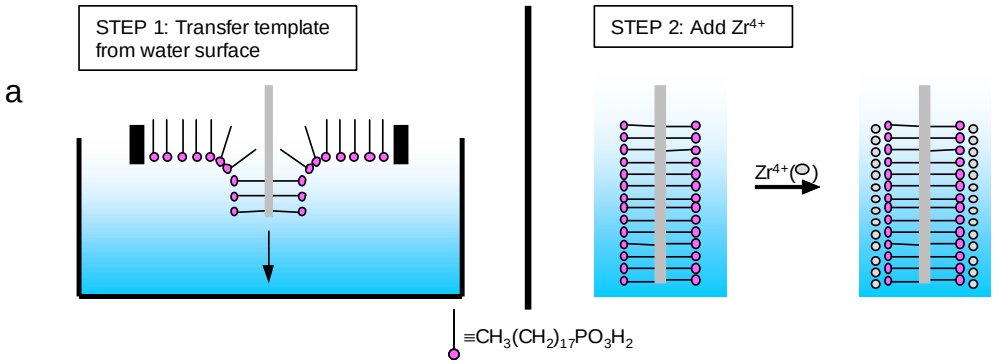
Adsorption
probe
specific
anchoring
groups
Si
Si
surface modifiers

2


a
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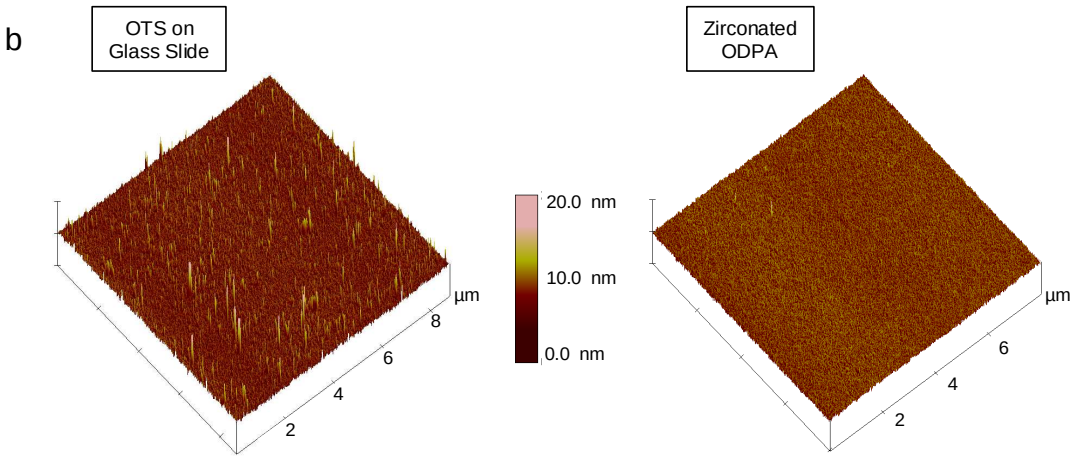
STEP 2: Add Zr^{4+}
Zr^{4+}(O)
$\equiv CH_3(CH_2)_{17}PO_3H_2$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OTS on Glass Slide
Zirconated ODPA
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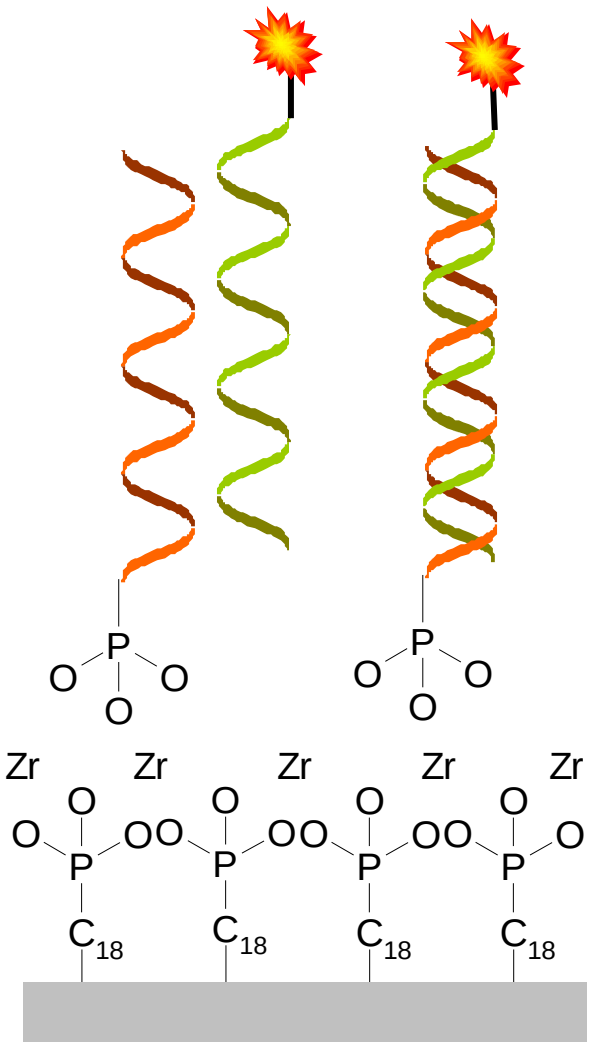
10.0 nm
0.0 nm
µm
2
4
6
8



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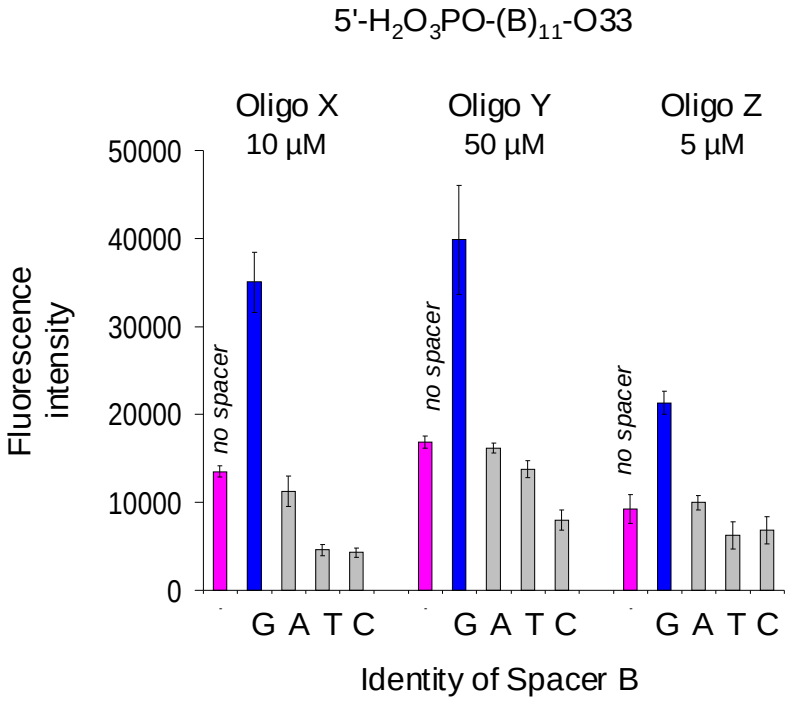
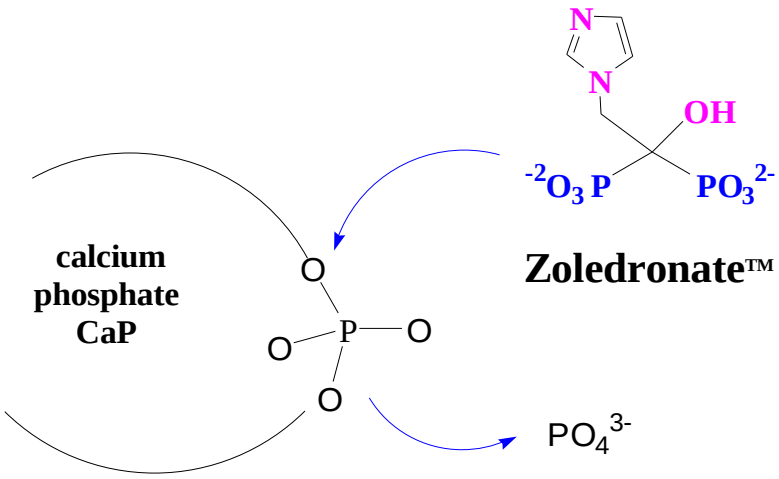

5'-H2O3PO-(B)11-O33
Oligo X
10 µM
Oligo Y
50 µM
Oligo Z
5 µM
50000
40000
30000
20000
10000
0
Fluorescence intensity
no spacer
no spacer
no spacer
G A T C
G A T C
G A T C
Identity of Spacer B

5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N
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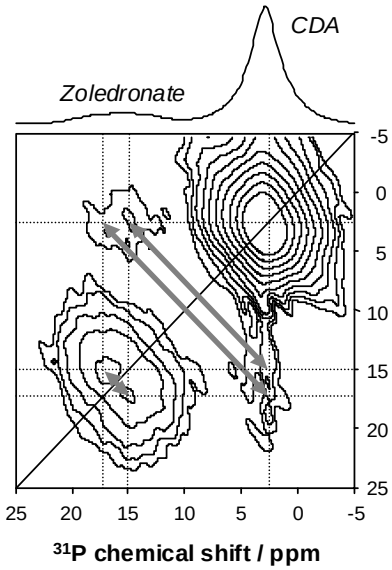
phosphate
CaP
O
P
O
O
O
PO_4^{3-}


CDA
Zoledronate
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10
15
20
25
25
20
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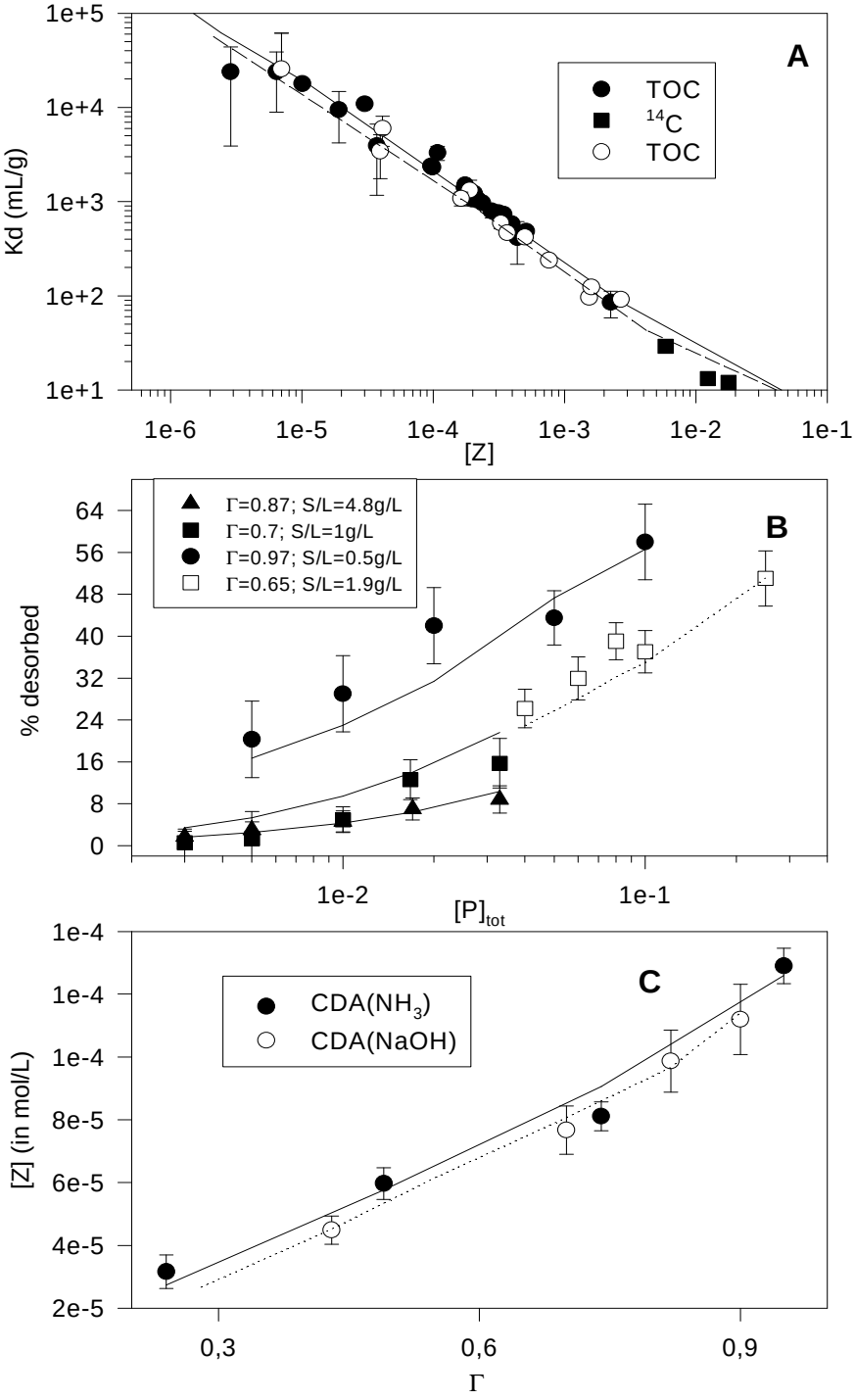
^{31}P chemical shift / ppm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TOC
14C
TOC
Kd (mL/g)
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1e+4
1e+3
1e+2
1e+1
1e-6
1e-5
1e-4
1e-3
1e-2
1e-1
[Z]
B
Γ=0.87; S/L=4.8g/L
Γ=0.7; S/L=1g/L
Γ=0.97; S/L=0.5g/L
Γ=0.65; S/L=1.9g/L
% desorbed
64
56
48
40
32
24
16
8
0
1e-2
1e-1
[P]tot
C
CDA(NH3)
CDA(NaOH)
[Z] (in mol/L)
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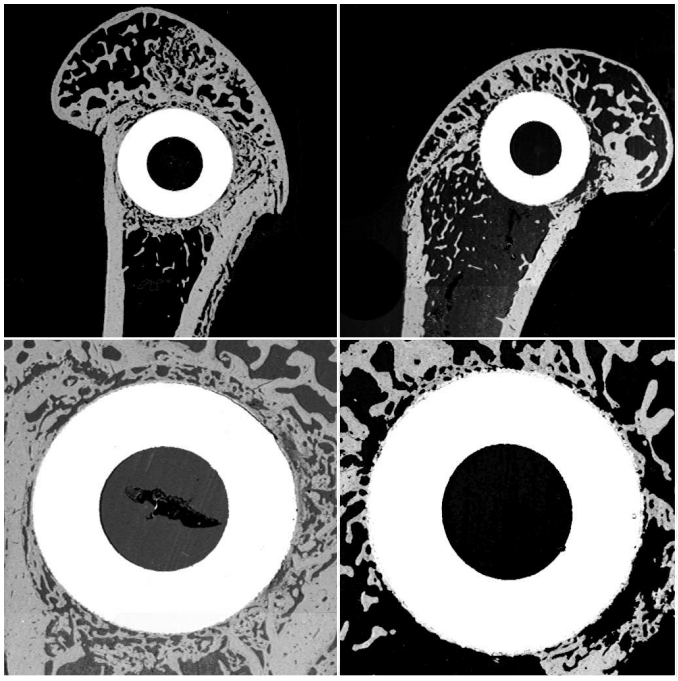
0,6
0,9
Γ

(a) **(b)**



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